

UNITED STATES DEPARTMENT OF AGRICULTURE
ANIMAL AND PLANT HEALTH INSPECTION SERVICE
VETERINARY SERVICES LABORATORIES
Post Office Box 844
Ames, Iowa 50010

DRAFT

SAM 125

9 CFR 113.XXX
Standard Requirement

September 1, 1995
New

Bovine Rotavirus
Agent

SUPPLEMENTAL ASSAY METHOD

FOR

TITRATION OF BOVINE ROTAVIRUS IN VACCINES

A. SUMMARY

This is an in vitro assay method which employs a cell culture system and cytopathic effect (CPE) or, alternatively, indirect immunofluorescence (IIF) to determine the Group A bovine rotavirus content of vaccines.

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B. MATERIALS

1. Cell Cultures

Multiple 96-well disposable plates are seeded (0.2 ml/well) with rhesus monkey kidney (MA-104) cells. Cells must be free of extraneous agents. The cells are seeded at a density that will produce 90-100% confluency after two days of incubation.

2. Growth Medium

The cells are grown in Minimum Essential Medium (MEM) with 7% fetal bovine serum and additives (Appendix, 1.) at a temperature of 35 to 37°C in an incubator containing an atmosphere of 5% carbon dioxide (CO₂) and a relative humidity of 70 to 80%. Growth medium is not changed unless excess acidity occurs or cells are not growing well.

3. Maintenance Medium

Maintenance medium (Appendix, 2.) without serum is used to rinse the cells prior to inoculation. It is also used as a diluent, in the presence of pancreatin*, for the virus titration assay.

* Pancreatin 4XNF (10X), Gibco Laboratories, catalogue no. 610-5720AG. No endorsement expressed or implied.

9 CFR 113.XXX
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4. Reference Virus

The National Veterinary Services Laboratories (NVSL) reference bovine rotaviruses for serotypes 6 (NCDV-Lincoln strain) and 10 (B223 strain) are used as controls for the cell system.

5. Primary Antibody

When indirect immunofluorescence (IIF) and not cytopathic effect (CPE) is used to titer the virus, serotype- or strain-specific antisera or monoclonal antibodies are used as primary antibody.

6. Fluorescent Antibody Conjugate

NVSL reference fluorescein isothiocyanate-conjugated immunoglobulin-specific antiserum is used in the IIF assay.

C. METHOD

1. Cells that have been seeded in 96-well plates four to six days previously are inverted and the growth medium removed by gentle shaking and tamping on sterile gauze. The cells are rinsed with 0.2 ml of maintenance medium per well, the medium again decanted from the plate, and the cells refed with 0.2 ml of maintenance media which remains on the plate for one hour at 37°C.

2. When a vaccine product is to be assayed, it is rehydrated with its accompanying diluent. Serial ten-fold dilutions are made of the bovine rotavirus samples to be tested, using maintenance medium containing a previously titrated amount of pancreatin, the maximum that the MA-104 cells will tolerate.

3. The final rinse is removed from the cells, as above. Each well is inoculated with 0.2 ml per well of each virus dilution, at a minimum of four wells per dilution. A minimum of eight wells remain uninoculated with virus, to serve as negative cell controls; they receive 0.2 ml per well of only the pancreatin-containing diluent.

4. The plates are incubated at 37°C, in an atmosphere of 5.0% CO₂ and high humidity, for five days. After five days, the cells can be examined for CPE typical of bovine rotavirus. The TCID₅₀ of each virus sample is calculated by the method of Spearman and Karber. The cells in the negative control wells must remain normal.

5. Certain strains of bovine rotavirus may not exhibit pronounced CPE, thus an IIF assay may be necessary to determine their titer.

a. After the medium is decanted, the cells are gently rinsed in phosphate buffered saline (PBS), then in deionized water. The cells are fixed in a solution of 80% acetone-20% deionized water at 4°C for 15 minutes. The acetone is discarded and the plates air-dried.

SAM 125

9 CFR 113.XXX
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b. The wells are covered with 0.1 ml per well of a previously-titrated dilution of specific primary antibody and held in a high humidity, 37°C incubator for 30 minutes. Excess primary antibody is washed from the plates by two gentle PBS rinses and one deionized water rinse. The plates are shaken gently and lightly touched to an absorbent towel to remove excess moisture.

c. While still moist, wells are covered with 0.1 ml per well of species-specific, conjugated anti-immunoglobulin antiserum. Again, the plates are incubated at 37°C for 30 minutes. Washing is repeated, as in step b. The plates are air-dried.

d. The cell monolayers are examined by fluorescent microscopy using a Ploem illuminator and blue light (Xenon lamp). Any cells showing immunofluorescence characteristic of bovine rotavirus are considered positive. The TCID₅₀ is calculated using the Spearman-Kärber method. For the test to be valid, the non-inoculated wells must be negative for immunofluorescence.

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Appendix

1. Growth Medium

MEM (Eagle) with Earle's salts*	1.0 packet
Deionized water q.s.	1.0 liter
Sodium bicarbonate	2.2 gram
Gentamicin sulfate	50.0 mg
Penicillin	25,000 U
Streptomycin	100.0 mg
Heat inactivated or irradiated fetal bovine serum	70.0 ml
200 mM L-Glutamine (100X)	292.0 mg
0.22 micron filtration	

2. Maintenance Medium

MEM (Eagle) with Earle's salts*	1.0 packet
Deionized water q.s.	1.0 liter
Sodium bicarbonate	2.2 gram
Gentamicin sulfate	50.0 mg
Amphotericin B	5.0 mg
Penicillin	100,000 U
Streptomycin	100.0 mg
200 mM L-Glutamine (100X)	292.0 mg
0.22 micron filtration	

* MEM with Earle's salts, Gibco Laboratories, catalogue no. 410-1500EB. No endorsement expressed or implied.